Standard Artifacts to Correct for Inherent Fluorometer Instrument-Associated Signal Detection Fluctuation

NIST is developing Standard Reference Material (SRM) glass artifacts to enable better fluorescence measurements in fields such as biotechnology, clinical diagnostics and drug discovery. Fluorescent probes are used to measure proteins and cells. Unlike with other physical measurement methods, most fluorometers lack the capability of correcting for individual instrument-related signal detection fluctuation. The new SRMs are UV-transmitting, high-durability, phosphate glass that can be used by scientists to calibrate their instruments to enable more accurate fluorescence measurements.

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Fluorescence measurements are single beam, and therefore, lack automatic ratioing method that removes the instrument function. Consequently, fluorometers measure sample fluorescence convolved with the instrument function of the device. If uncorrected, such results can be misleading, especially at the short wavelength end where the excitation source is weak and at the long wavelength end where the detector response is dying; but even in the mid-range, bumps and dips show up that are due to the wavelength selector (monochromator) and detector responses, not to the native fluorescence of the sample.

We have recently completed the certification of SRMs 2940 and 2041, Relative Intensity Correction Standards for Fluorescence Spectroscopy, with orange emission and green emission respectively. These fluorescent glass artifacts are the first new fluorescence spectral correction CRMs issued by an NMI in 25 years (NIST certified SRM 936, Quinine Sulfate Dihydrate in 1979 – R. Velapoldi and K. Mielenz). We plan to produce a series of such standards for use from the near ultraviolet to the near infrared. The orange and green emitting standards were produced in a borate glass; however, this matrix is not suitable for ultraviolet/violet and blue SRMs because it does not transmit well in the ultraviolet region. Accordingly, we began a search for a suitable glass matrix. The ideal glass should be transparent far into the ultraviolet, should be rugged and resistant to corrosion from handling and atmospheric humidity, should be a good solvent for the inorganic metal oxide fluorophor dopants, and should melt at a reasonable temperature with a low viscosity so that bubble entrapment would not be a problem. For easy production, the ideal glass should be resistant to devitrification, anneal easily, have a sufficiently low coefficient of expansion to avoid cracking during production, and be hard enough to polish easily.

NIST's new SRM glass artifacts will enable truer quantitation of proteins and other biomolecules and cells when measured with fluorescent probes.

To develop suitable glasses, we set up a small melting and fabrication facility where we can produce test melts on a small (50 g) scale as well as cutting and polishing the glass into prototypes for testing. In doing this, we were both mentored and encouraged by folks who really know glass. Without the assistance of Doug Blackburn and Wolfgang Haller (both retired from the old glass group in MSEL), from Jack Fuller and Jeff Anderson in the NIST Optical and Glass Shops, and from Joe Hayden at Schott Glass, this project could not have been accomplished.

From the literature, we assembled a list of glass types with acceptable properties that we discussed with our mentors, who immediately eliminated many of the possibilities. Other candidates were ruled out because our melt oven was then limited to 1200 ˚C. We eventually discovered a calcium alumino phosphate glass that seemed to have what we wanted. However, there was a problem, most known fluorescing metal oxide dopants failed to produce fluorescence in our glass. This turned out to be caused by impurities from the ingredients used to make the glass and from the inexpensive silica/alumina crucibles we were then using. Switching to high purity alumina crucibles solved most of the problem, and a study of ingredients from several vendors led us to sources of suitable ingredients.

The basic formulation for our glass is 47 mole % CaO, 3 mole % Al_2O_3, and 50 mole % P_2O_5 (the dopant concentrations is <0.01 mole %). The precise ingredients used for a given batch depend upon the redox chemistry we are trying to carry out. Many of the metal oxide dopants we are studying exist in multiple oxidation states, not all of which fluoresce. The desired oxidation state in the glass is achieved by controlling the local atmosphere in and around the glass as it is melted, through the choice of ingredients and/or by carrying out the melt in a suitable gaseous atmosphere. For example, copper I ions in glass are fluorescent, while copper II ions are not. Doping with copper I oxide is not sufficient to ensure that the copper will remain in that state in the glass. To produce copper I in glass, reducing melt conditions are employed. Our best results with copper have come by supplying some of the phosphate from ammonium dihydrogen phosphate (at 1200 ˚C the liberated ammonia burns and consumes the local oxygen) in a forming gas (5 % H_2 in N_2) atmosphere. The resulting glass is colorless and fluorescent (copper II glass is the familiar blue color and does not fluoresce in the uv/vis region).

Most phosphate glasses have bad reputations for stability. Many (especially those with Group I cations) are rather soft and corrode easily in humid air. Phosphate glasses with Group II cations are more resistant to water damage. The addition of a small amount (<8 mole %) of alumina) has been shown to improve both the hardness and corrosion resistance. A DI water corrosion test showed our glass to be only an order of magnitude less stable than ordinary window glass. A similar study on the borate glasses used to make SRM 2940 and SRM 2041 showed them to be almost an order of magnitude more easily corroded that our phosphate glass.

The uv/visible spectrum of our calcium alumino phosphate glass exhibits transmittance down to 220 nm, which is better than some quartz or fused-silica materials. However, to achieve this performance, the ingredients and the crucibles must be essentially free of metallic impurities, especially iron. Just a few µg/g can dramatically raise the uv cut-off wavelength. The glass shows good solubility for most metal oxides, and we have used it to make over 30 different metal-oxide-doped fluorescent glasses and now have several SRM candidates that we are testing for photostability. Once we select the most promising glasses, we will make production batches in the MSEL glass-making facility and send them to Optiglass, Ltd. for cutting and polishing prior to certification.

The discovery of the availability of calcium alumino phosphate glass will enable us to produce:

SRM 2942 (blue emission),
SRM 2943 (uv/violet emission)

it also looks very promising as a matrix for:

SRM 2944 (red emission)
SRM 2945 (nir emission).

CSTL researchers J. Seiber and A. Marlow performed the necessary X-ray fluorescence analyses for this work.